

A COMPARATIVE STUDY OF THE ACCURATE MEASUREMENT OF ENDOTOXIN IN LIPOSOME ENCAPSULATED HEMOGLOBIN

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ABSTRACT

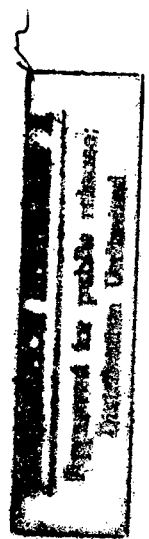
We have examined three different methods of endotoxin determination utilizing the Limulus Amebocyte Lysate (LAL) assay to accurately determine endotoxin levels in Liposome Encapsulated Hemoglobin (LEH), 1) the gel-clot method, 2) chromogenic spectroscopic-based LAL, and 3) the turbidimetric method which determines endotoxin levels in solutions based on the time needed to reach a specific degree of turbidity. Both the chromogenic and turbidimetric methods require significant dilution of the LEH preparation before accurate measurement can be made. We have tested the levels of endotoxin in LEH solutions using these methods and measured LEH, liposome, and hemoglobin samples spiked with known amounts of endotoxin. A comparison of the three methods shows that the absolute value of endotoxin measured in LEH by the three methods can vary significantly. However, within any one assay the spiked amount of endotoxin in the sample can be accurately measured. The accuracy of these methods may also be complicated by the binding of endotoxin to LEH. This was evident by mixing free endotoxin with LEH followed by centrifugation to separate the LEH. Biological activity of endotoxin bound to LEH was measured by exposure to RAW264.7 followed by the expression of tumor necrosis factor.

INTRODUCTION

Hemorrhage has been shown to induce significant immunomodulation and transitory depression of reticulo-endothelial system (RES) function (1). Consequently, the administration of hemoglobin-based blood substitutes has been implicated in the exacerbation of septic shock (2). This is especially relevant for LEH which has been shown to accumulate in organs of the RES (3). We have undertaken the present studies to define the interaction of LEH with lipopolysaccharide (LPS) which has been used to mimic the initial phases of sepsis. Previous studies have suggested that hemoglobin binds endotoxin (4). In addition, as LPS is an amphiphile, the lipid A portion can be incorporated into lipid bilayers while free LPS can be encapsulated within liposomes, both of which reduce the biological activity of LPS (5). These studies also have significance with relation to manufacture of LEH and quality control of LEH solutions at the bench.

In December 1987 the FDA published guidelines for the validation of the LAL test as an alternative to the USP Rabbit Pyrogen Test for end-product testing of biological products, human

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and animal parenteral drugs, and medical devices (6). The LAL test was developed based on the observation by Bang that Gram-negative infection of *Limulus polyphemus*, the horseshoe crab, resulted in fatal intravascular coagulation (7). Bang and Levin determined that the clotting was due to the lipid A portion in endotoxin from the bacteria, and a clottable protein in the circulating amebocytes of the *Limulus* (8). The mechanism by which this clotting reaction occurs is enzymatic and involves the catalytic activation of proenzyme by endotoxin to the enzyme coagulase, which then hydrolyses the clotting protein coagulogen with a resultant self-associated clot of coagulin (9).

We have utilized three forms of the LAL test in our assessment of endotoxin concentrations in LEH preparations; the gel-clot method, the turbidimetric method and the chromogenic method. Each method relies on the formation of the activated enzyme. In the gel-clot method the sample may form a clot which is determined by inversion of the sample and macroscopic gel formation. If the sample clots there is at least as much endotoxin present as the lysate degree of sensitivity. Endotoxin levels are determined over a range based on serial dilutions of the sample. The principle of the turbidimetric method is based on the time necessary for a sample to reach a specific degree of turbidity based on the initiation of the clotting reaction. We have used two chromogenic methods, one method utilizes the activated enzyme to catalyze the splitting of p-nitroaniline (pNA) from a colorless substrate, the pNA is measured spectrophotometrically at 405-410 nm with a sensitivity of 0.1-1.0 EU/ml. The other method has increased sensitivity (0.015 to 0.25 EU/ml) by reacting the released pNA with nitrite in HCl and then with N-(1-Naphthyl)-ethylenediamine (NEDA) to form a diazole derivative that absorbs between 540-550 nm. An advantage of this method is that the spectral absorbance is measured at wavelengths away from the solet band which decreases the background interference of hemoglobin samples.

MATERIALS AND METHODS

LEH was prepared by sterile technique as described previously. (10). In brief, purified bovine hemoglobin (Biopure) was added to a lyophilized mixture of distearoyl phosphatidylcholine (Avanti Polar Lipids), cholesterol (Calbiochem), dimyristoyl phosphatidylglycerol (Avanti), and vitamin E (Aldrich) at a molar ratio of 10:9:0.9:0.1. After 24-hour hydration, the mixture was processed to a more homogeneous particle size dispersion by microfluidization (Microfluidics). Non-encapsulated hemoglobin was removed by centrifugation, and the pelleted LEH was suspended in Dulbecco's PBS (ICN Flow) to a hemocrit of approximately 40%. The LEH was tested for liposome size, phospholipid concentration, total hemoglobin, percent methemoglobin, oxygen-binding capacity and sterility. The LEH used in these experiments was sterile and of consistent characteristic profiles with previously produced LEH preparations. LPS (*E. coli* 0113:H10, Associates of Cape Cod, Woods Hole, MA) was diluted from a 100 EU/ml stock solution for the spiking experiments and from a 5 mg/ml (*E. coli* 0111:B4 Calbiochem, La Jolla, CA) stock solution for cell culture experiments. All glassware used was depyrogenated by baking for four hours at 180°C, plasticware was autoclave sterilized.

Each of the LAL methods used are available commercially in kit form. Each method was performed in accordance with the protocol provided with each kit. The gel-clot method was tested using the Endotect™ Gel Clot Kit (ICN, Costa Mesa, CA). Serial dilutions of LEH are pulled into Endotect capillary tubes that contain lysate and then dry incubated at 37°C for one hour. After one hour the tubes are removed and inverted to observe the formation of a clot. The sensitivity of the lysate used in this method was 0.06 to 0.1 ng/ml. One of the chromogenic methods used was the

QCL-1000 Quantitative Chromogenic LAL (BioWhittaker, Walkersville, MD). Standards and samples are mixed with lysate in a 96-well pyrogen-free plate (ICN Flow, CA) and incubated at 37°C for 10 minutes. A chromogenic substrate solution is then added followed by a 6 minute incubation period. The reaction is stopped with 10% SDS and the pNA absorbance is read at 405-410 nm on a spectrophotometric plate reader (Beckman Biomek). A linear correlation exists between the absorbance and the endotoxin concentration for standards from 0.1 to 1.0 EU/ml. The determination of endotoxin in samples is evaluated along the standard curve using curve-fitting software (TableCurve, Jandel Scientific, CA).

The other chromogenic method used in this study was the LAL Pyrochrome™ (Associates of Cape Cod, Woods Hole, MA). Samples and standards were added to the inside wells of the 96 well supplied with the kit. An equal volume of Pyrochrome LAL was rapidly added to each sample and mixed on a shaker for 30 seconds. Following 30 minute incubation at 37°C, equal volumes of mixed sodium nitrite in HCl, ammonium sulfamate and NEDA were added. Full color development occurs within 1 to 3 minutes, at which time the absorbance at 540-550 nm was measured. The chromogenic determination with diazo-coupling has a standard curve sensitivity from 0.0156 to 0.25 EU/ml. The turbidimetric method was performed with the LAL-5000 (Associates of Cape Cod, Woods Hole, MA). This instrument measures the turbidity of an LAL/standard or sample mixture at regular intervals. The system contains software that analyzes the stored data and determines the time taken for the optical density to reach a fixed threshold level. This time, called the onset time, decreases with higher endotoxin concentrations. The log of the endotoxin concentration is inversely proportional to the log of the onset time. The LAL-5000 quantifies the concentration of endotoxin present in samples by evaluation against standard curves generated using known quantities of standard endotoxin, the detection range over which is 0.001 to 100 EU/ml. In each of the chromogenic and the turbidimetric methods, LEH samples had to be diluted to at least 1:200 in pyrogen-free water to eliminate background interference from light scattering of the liposomes in the turbidimetric method, and the absorbance of hemoglobin in each of the spectrophotometric methods.

In order to investigate the binding of LEH and LPS, 0.1% LEH (by volume) was mixed with 1.0 µg LPS for 1 hour at 37°C in depyrogenated glassware. Following the incubation period, the suspension was centrifuged and the LEH pellet washed three times with cell culture media (DMEM). The LEH pellet was then resuspended and aliquoted into 24 cell well plates containing RAW 264.7 cells. Cells were grown in DMEM supplemented with 10% FBS, then plated at 5×10^5 cells per well. The media over the cells was collected at 1, 2, 4, 6, and 24 hours after incubation with LEH. LEH not exposed to LPS, and LPS alone were run as negative and positive controls, respectively. The quantification of TNF- α was performed utilizing an ELISA (Genzyme, Cambridge MA).

RESULTS

Table 1 shows the results in comparing endotoxin detection by the chromogenic method to the turbidimetric method. In comparison, LEH and liposomes measured turbidimetrically displayed values 3-5 times those obtained by the chromogenic method. Endotoxin values measured by these two methods for free hemoglobin were similar. The levels of spiked endotoxin in the LEH samples were accurately measurable by both methods. When standards for the chromogenic method were treated as samples in the turbidimetric method, in we found the two methods to be internally consistent but without correlation to each other (data not shown).

Table 1

Sample	Chromogent (EU/ml)	Turbidimetric (EU/ml)
LEH	8.5	21.5
Liposomes	5.6	34.0
Hemoglobin	1.9	1.8
LPS 2ng	60.0	18.5
LEH + LPS 2ng	66	32.7
Liposomes + LPS 2ng	62.3	35.2
Hemoglobin +LPS 2ng	108.0	41.1

Table 2 shows the comparison of detection by the Chromogent QCL to the Pyrochrome method (samples diluted as shown). The chromogenic method requires significant background subtraction to remove the absorbance due to hemoglobin which resulted in loss of consistency and accuracy. The pyrochrome method required no background subtraction at similar dilutions and demonstrated consistent endotoxin values at each dilution.

Table 2

LEH Dilution Factor	Chromogent EU/ml	Pyrochrome EU/ml
1/200	0	2.3
1/400	7.6	4.3
1/800	0	5.5

Table 3 shows the results of LEH samples spiked with endotoxin at 2 concentrations as detected by the Pyrochrome method. Spike samples were evaluated at different dilutions. The results demonstrate that the spiked levels of endotoxin in LEH solutions can be accurately detected by this method with good consistency at different dilutions.

Table 3

LEH Dilution Factor	EU/ml
Low Spike (5EU/ml)	
1/200	4.8
1/400	5.4
1/800	7.7
High Spike (20EU/ml)	
1/200	16.8
1/400	16.1
1/800	16.0

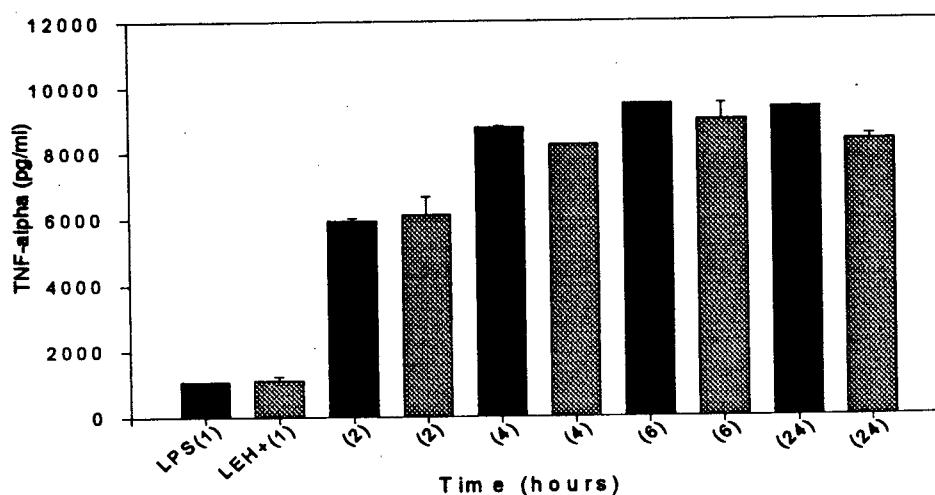
Semi-quantitative values were determined for endotoxin values using the terminal gel clot in accordance with the protocol and ranges of concentrations were determined as shown in Table 4.

Table 4

Sample	Result
Negative control	<0.1 ng/ml
Positive control	>0.1 ng/ml
LEH	<20 ng/ml
LEH Spiked with 5 EU/ml	>20 ng/ml

Perhaps one reason for the inconsistency of endotoxin measurement between assays is the interaction of endotoxin and LEH. Figure 1 shows the results from an ELISA for TNF- α from samples of LEH that had been incubated with 1.0 μ g LPS. LEH alone does not induce TNF- α production, however LEH co-incubated with LPS and then separated by centrifugation showed TNF- α levels comparable to LPS alone by 4 hours. This indicates that endotoxin may bind to LEH and that the bound endotoxin remains biologically active.

Figure 1



DISCUSSION

The ability to detect endotoxin in LEH by LAL has been examined. The particulate suspension of LEH and the turbid nature of the samples brings up a critical issue of whether LEH interferes with the gelation based LAL reaction. Dilutions of LEH solutions indicate that the most consistent assay for determination of endotoxin was the chromogenic based pyrochrome. This assay also was read at an absorbance away from any interference from hemoglobin. Due to its ease of use, the gel-clot may be used as a pre-screening assay to determine the range of endotoxin in LEH samples. Both the turbidimetric and Pyrochrome methods were superior in determining endotoxin levels that had been spiked into LEH.

In order to address the potential immunomodulation of LEH as it accumulates in the reticuloendothelial system, it is of extreme importance to have definitive endotoxin concentration determinations. The potential interaction of LPS and LEH also complicates both the accurate

measurement of endotoxin and the potential effects of LEH in combined conditions of hemorrhage and sepsis. We have presented preliminary data which shows that LEH may bind LPS and that the bound LPS is biologically active. This could result in changes in which endotoxin is presented in both the LAL test and in biological activities associated with endotoxin in vitro and in vivo models.

The ultimate accuracy of the LAL test for LEH solutions will require further validation. We have seen LEH preparations that have measured 5-10 EU/ml by the pyrochrome LAL test and have passed the USP rabbit pyrogen test. Future experiments include the use of radiolabeled LPS as a marker to further define the interaction between LEH and LPS as well as models which explore the biological activity of LEH solutions.

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